Inhibition of Glycolysis by Amino Acids in Ascites Tumor Cells

SPECIFICITY AND MECHANISM*

(Received for publication, July 23, 1992)

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The effect of glutamine and asparagine on glucose metabolism has been studied in ascites tumor cells. Either of these amino acids decreased the glycolytic flux about 80%. Half-maximal effects were obtained with 0.14 mM glutamine and 0.087 mM asparagine. Among the 20 l-amino acids, only glutamate produced a similar effect. Glutamine and asparagine caused a 70% increase of hexose monophosphates and a large decrease of fructose-1,6-P₂ and triose phosphates, evidencing a strong inhibition of the phosphofructokinase (EC 2.7.1.11) reaction. Analysis of the levels of various phosphofructokinase effectors revealed that fructose-2,6-P₂ and AMP decreased 4-fold, phosphoenolpyruvate, citrate, and ATP increased 4-, 3-, and 1.8-fold, respectively, and that there was no change in ADP, Pᵢ, and intracellular pH. Assay of phosphofructokinase at concentrations of substrates and effectors determined to be in the cells showed that the low activity of this enzyme could be accounted for by the change in the concentration of effectors, the major mechanism being the change in adenine nucleotides. The decrease in fructose-2,6-P₂ contributed very little to the inhibition of phosphofructokinase activity. The effects of amino acids were prevented by amino-oxyacetate, suggesting that transamination was an obligatory step for these changes.

Many tumors and rapidly dividing cells in general exhibit a characteristically enhanced aerobic glycolysis (1, 2). The mechanism and significance of this observation remain unclear despite numerous explanations that have been advanced to account for it (1–9). This type of cell also exhibits a remarkably high utilization of glutamine, which serves both as a precursor in the synthesis of nucleotides, proteins, and other compounds and as a major energy substrate (1, 2, 10). In Ehrlich ascites cells, glutamine is oxidized 20 times faster than glucose (11) with production of glutamate, aspartate, and CO₂ (12). The pathways of glutamine metabolism have also been investigated in isolated mitochondria (13, 14). Asparagine is another amino acid for which malignant cells show a marked preference, and this is a basis for the antitumor activity of asparaginase (15, 16), which depletes circulating asparaginase in animals and cancer patients (17, 18). Nevertheless, the significance of the great demand for amino acids shown by proliferating cells is still poorly understood (1, 10, 14, 19). It has been suggested that high rates of glycolysis and glutamine degradation are required for high sensitivity of the pathways to specific regulators (2). However, other authors (1) have questioned a simultaneous flux through both processes as essential for cell proliferation on the basis of glucose limitation experiments and the use of mutants with defects in their oxidative metabolism.

Glutamine has been reported to decrease the levels of fructose-2,6-P₂ in HeLa cells and chick embryo fibroblasts (20), producing a mild diminution of the glycolytic flux only in the latter type of cells. Addition of glutamine to L929 cell cultures with glucose and insulin has been shown to stimulate lactate production (21) without change in glucose oxidation, but the mechanism has not been clarified. Therefore, we reinvestigated the possibility of a significant interaction between the metabolism of glutamine and asparagine and the control of glucose consumption in cancer cells. We have examined whether these amino acids affect the concentration of fructose-2,6-P₂ and the operation of glycolysis, as well as their influence on other signals considered important for the regulation of this pathway. Ascites tumor cells were chosen since it is well known that they show high aerobic glycolysis (11, 22) and a great dependence on glutamine (11–14) and asparagine (18, 23). We found that these amino acids strongly inhibit the glycolytic rate and that this inhibition is due to an intense decrease in the flux through the phosphofructokinase reaction. Elucidation of the specificity and mechanism of this phenomenon is also reported.

MATERIALS AND METHODS

Chemicals and Enzymes—Phosphoric esters, nucleotides, PEG,¹ phenylmethylsulfonyl fluoride, leupeptin, cycloheximide, and auxiliary enzymes were purchased from Sigma. Radiochemicals were obtained from Amersham International. Amino acids were also from Sigma except for aspartate, glutamine, and proline, as well as other chemicals, that were obtained from Merck.

Ehrlich Ascites Cells—A hyperdiploid strain of Ehrlich-Lettre ascites carcinoma cells was maintained by weekly inoculation into the abdominal cavity of 2-month-old male Swiss mice. The animals received standard Panlab food (Barcelona, Spain) and water ad libitum. Cells were harvested 7–9 days after inoculation, suspended in 38.5 mM NaCl, centrifuged at 1,000 × g for 5 min at room temperature.

† This work was supported by Grant PB89–0168 from the Dirección General de Investigación Científica y Técnica. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.


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1The abbreviations used are: PEG, polyethylene glycol; TES, Tris(hydroxymethyl)aminomethane; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); PIPES; piperazine-N,N'-bis(2-ethanesulfonic acid); P-enolpyruvate; phosphoenolpyruvate.
temperature, twice washed free of erythrocytes in 115 mM NaCl, 5 mM potassium phosphate, pH 7.4, and resuspended to 25 mg/ml (wet weight) in buffer A (50 mM TES, 50 mM PIPES, 85 mM NaCl, 6 mM KCl, pH 7.0). Five to seven animals were used for each experiment.

**Incubations** — The cell suspension was preincubated for 90 min under aerobic conditions at 37 °C, to deplete the intracellular pool of endogenous metabolites and to place cells coming from different animals in a similar situation. At the end of the preincubation, cells were collected by centrifugation. A gram of wet weight under these conditions, considered throughout this work as the reference value, is equivalent to (0.50 ± 0.02) × 10⁻⁷ cells. The cells were suspended to 40 mg/ml in buffer A and distributed in aliquots. Additions indicated in each experiment were made and the cells incubated for various periods of time under aerobic conditions, at 37 °C, with gentle shaking. More than 90% of the cells remained viable after incubation for 180 min, as estimated by trypan blue staining. Incubations were terminated by addition of 8% (v/v) perchloric acid in 40% (v/v) ethanol unless otherwise indicated.

**Metabolite Measurements** — For the measurement of fructose-2,6-P₂, samples of 1 ml of cell incubations were centrifuged at 1,000 × g for 30 s. The cell pellets were deproteinized at 80 °C for 10 min with 0.4 ml of hot (80 °C) 0.1 M NaOH. After homogenization and centrifugation at 25,000 × g for 10 min, the supernatant was assayed for fructose-2,6-P₂ as a stimulator of potato pyrophosphatase:fructose-6-phosphate phosphotransferase (24). This stimulation was suppressed by treatment of the extract at 25 °C at pH 1-2 for 10 min. For the measurement of glycolytic intermediates, citrate, ATP, ADP, and inorganic phosphate concentrations of cell supernatants were determined in 1 ml of ice-cold 4% (v/v) perchloric acid in 40% (v/v) ethanol. After centrifugation at 25,000 × g for 10 min, the supernatant was neutralized to pH 7.0 with a solution containing 0.5 M KOH and 0.5 M triethanolamine, the mixture was allowed to stand on ice for 15 min, and precipitated KClO₄ was removed by centrifugation. Metabolites were measured by standardized extraction techniques (25, 26) in a Shimadzu model UV3000 dual wavelength spectrophotometer, set to read absorbance at 340 nm minus 400 nm. For the measurement of AMP, samples of 5 ml of cell incubations were centrifuged at 1,000 × g for 30 s, and the cell pellets were deproteinized with 1 ml of 10% (v/v) perchloric acid in 40% (v/v) ethanol as above. AMP was determined as described (27). The ATP used in the assay was purified by chromatography on Dowex-1 chloride (X2) to remove nucleotide impurities. Inorganic phosphate was measured using an enzymatic method similar to that reported by Guynn et al. (28), except that hexokinase was not included in the assay.

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The intracellular water volume was determined by using [³H]H₂O and [¹⁴C]uridine following a procedure similar to that of Rottenberg (29). The intracellular water thus obtained was 56 ± 2%. A wet weight/dry weight ratio of 4.6:1 was used to calculate the intracellular concentration of metabolites and to distribute the internal water volume. The precipitate was collected, dissolved in buffer A, and filtered through a column of medium Sephadex G-25 (0.5 × 22 cm) equilibrated and eluted with the same buffer. This preparation had a specific activity of 15% (v/v) dissolved in 10 mM HEPES, 5 mM EDTA, pH 7.0, containing 20% (v/v) glycerol (0.5 ml/g of cells) and filtered through a column of medium Sephadex G-25 (0.5 × 22 cm) equilibrated and eluted with the same buffer. This preparation had a specific activity of 3:1.

**Enzyme Assays** — Phosphofructokinase activity was measured in an assay mixture that unless otherwise indicated contained 50 mM HEPES, 0.1 M KCl, 5 mM MgCl₂, pH 7.2, 0.15 mM NADH, 2.8 mM MgATP, 1.2 units of aldolase, 10 units of triosephosphate isomerase, 1 unit of glyceraldehyde-3-phosphate dehydrogenase, and 10 μl of the enzyme preparation in a total volume of 400 μl. After incubation for 10 min at 37 °C, the reaction was terminated by addition of 100 μl of 0.1 M NaOH followed by heating at 80 °C. After cooling on ice, the mixture was centrifuged for 10 min at 15,000 × g and the supernatant assayed for fructose-2,6-P₂ as described above. Under these conditions, the production of fructose-2,6-P₂ was linear for at least 20 min. Glucose-6-P was always added to fructose-2,6-P₂ in the proportion 3:1.

**Fructose-2,6-bisphosphatase activity was measured by following the disappearance of fructose-2,6-P₂. Unless otherwise indicated, the assay mixture contained 50 mM HEPES, 0.1 M KCl, 10 mM MgCl₂, pH 7.0, 1 mM Pi, 1 mM fructose-6-P, 1 mM MgATP, and 10 μl of the enzyme preparation in a total volume of 400 μl. After incubation for 10 min at 37 °C, the reaction was terminated by addition of 100 μl of 0.1 M NaOH followed by heating at 80 °C, and the remaining fructose-2,6-P₂ was measured as above. Under these conditions, the disappearance of fructose-2,6-P₂ was linear for at least 5 h.

**Other Assays** — Intracellular pH was measured in samples of cell incubations by determining the distribution of [¹³C]benzoic acid essentially as described by Doppler et al. (30). Protein was determined by the method of Bradford (31).

**RESULTS**

**Effect of Certain Amino Acids on Fructose-2,6-P₂ Content** — Fructose-2,6-P₂ is the most potent effector of glycolysis because of the strong allosteric activation exerted on phosphofructokinase (32). Therefore, the first step in our investigation was to ascertain whether glutamine and asparagine modified the levels of fructose-2,6-P₂. The content of this compound increased 27 times after incubation of tumor cells with 5 mM glucose for 20 min and then remained nearly constant (Fig. 1). Addition of 1 mM of either glutamine or asparagine reduced the content of fructose-2,6-P₂ by 70–80% after incubation for 180 min. The effect of the amino acid was apparent only after 20 min of incubation, when the levels of fructose-2,6-P₂ reached a maximum. A similar effect was observed when both amino acids were added together or when each of them was added after preincubation of tumor cells with glucose for 40 min (data not shown). As seen in Fig. 1, glutamate exhibited...
a pattern similar to that of glutamine, while aspartate had a much smaller effect.

Among the 20 L-amino acids, only isoleucine and proline produced an effect on the levels of fructose-2,6-P₂ similar to that of asparagine, glutamate, and glutamine (Table I). NH₄Cl was without effect, either alone or in the presence of aspartate. None of the amino acids that elicited a change in the content of fructose-2,6-P₂ modified the production of total lactate by tumor cells after incubation for 180 min. Addition of either asparagine, glutamate, or glutamine increased the content of hexose monophosphates by about 50%, suggesting a decrease in the flux through the phosphofructokinase reaction. However, no significant change in hexose monophosphates occurred with aspartate, isoleucine, and proline.

The decrease in fructose-2,6-P₂ was dose dependent, and the concentrations of amino acids required for half-maximal effect were 0.05 mM for proline, 0.07 mM for asparagine, 0.11 mM for isoleucine, 0.17 mM for glutamine, 0.35 mM for glutamate, and >1.0 mM for aspartate (data not shown). The concentrations of these amino acids in mouse plasma are within the range of values reported by others (11, 34). Neither glutamine, asparagine, nor proline, used at a concentration of 1 mM, significantly modified the time course of lactate production during incubation up to 180 min in the presence of sugar (data not shown). However, the observation that hexose monophosphates accumulated when glutamine or asparagine were added to the incubation medium (Table I) suggested a change in the glycolytic rate. Accordingly, we measured the release of labeled anions (mainly lactate and pyruvate) from [U-¹⁴C]glucose into the medium to assess if lactate derived from the amino acid may compensate for a decrease in lactate production from glucose. Fig. 2A shows that glutamine and asparagine diminished the production of labeled anions by about 45% after incubation for 180 min. The glycolytic flux progressively slowed after 60 min of incubation and was reduced by about 80% between 120 and 180 min (0.17 and 0.10 µmol of lactate plus pyruvate/min/g cells in the presence of glutamine and asparagine, respectively, versus 0.72 µmol/min/g cells in the control with glucose alone). The decrease in lactate production was paralleled by a practically stoichiometric reduction of [U-¹⁴C]glucose consumption under similar conditions (Fig. 2B). A similar diminution in glycolysis was observed when the inhibitory amino acid was added after preincubation of tumor cells with glucose for 60 min (data not shown), Fig. 2, A and B also shows that, in contrast to glutamine and asparagine, glucose metabolism was affected much less by proline.

The effect of glutamine and asparagine was concentration-

**Fig. 1. Effect of selected amino acids on content of fructose-2,6-P₂ of ascites tumor cells as a function of time.** Cells were incubated with 5 mM glucose without further addition (control conditions) or plus 1 mM amino acid as indicated. Each point shows the mean ± S.E. of three to five separate experiments. Differences between incubations with glucose and glucose plus amino acid that are statistically significant are indicated by * (p < 0.05), ** (p < 0.01). Only significance values corresponding to incubations for more than 30 min are shown to simplify the figure. When nearby points had the same significance value this is shown only once.

**Table I**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Fructose-2,6-P₂</th>
<th>Lactate</th>
<th>Fructose-6-P + glucose-6-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.6 ± 0.2 (7)</td>
<td>120.3 ± 6.4 (3)</td>
<td>0.17 ± 0.02 (6)</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.6 ± 0.1 (3)**</td>
<td>120.3 ± 3.6 (3)</td>
<td>0.26 ± 0.02 (6)**</td>
</tr>
<tr>
<td>Aspartate</td>
<td>2.0 ± 0.3 (9)</td>
<td>132.0 ± 1.3 (3)</td>
<td>0.18 ± 0.01 (3)</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>3.0 ± 0.4 (5)</td>
<td>132.0 ± 1.3 (3)</td>
<td>0.26 ± 0.02 (6)**</td>
</tr>
<tr>
<td>Aspartate + NH₄Cl</td>
<td>2.0 ± 0.1 (6)*</td>
<td>132.0 ± 1.3 (3)</td>
<td>0.27 ± 0.04 (6)*</td>
</tr>
<tr>
<td>Glutamate</td>
<td>0.7 ± 0.1 (4)**</td>
<td>137.9 ± 0.6 (3)</td>
<td>0.25 ± 0.04 (3)*</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.9 ± 0.2 (3)**</td>
<td>125.2 ± 2.0 (3)</td>
<td>0.21 ± 0.01 (3)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.9 ± 0.2 (3)**</td>
<td>125.2 ± 2.0 (3)</td>
<td>0.19 ± 0.03 (3)</td>
</tr>
<tr>
<td>Proline</td>
<td>0.7 ± 0.1 (5)**</td>
<td>125.2 ± 2.0 (3)</td>
<td>0.19 ± 0.03 (3)</td>
</tr>
<tr>
<td>All other amino acids</td>
<td>2.7 ± 0.1 (3-6)</td>
<td>125.2 ± 2.0 (3)</td>
<td>0.19 ± 0.03 (3)</td>
</tr>
</tbody>
</table>
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dependent. Half-maximal decrease in the rate of release of labeled anions, evaluated between 120 and 180 min incubations, was obtained with 0.14 mM glutamine and 0.087 mM asparagine (data not shown). This effect is relevant to the situation in vivo because these values are in the order of the physiological concentrations of these amino acids (33). Glutamate (1 mM) also decreased the release of labeled anions from glucose, after incubation of cells for 180 min to an extent similar to that of glutamine or asparagine, whereas all other amino acids used at the same concentration were without effect (data not shown). The influence of glutamine on glycolysis was not modified by 0.2 mg/ml cycloheximide (data not shown).

Changes in key glycolytic intermediates during the inhibition of the pathway are shown in Fig. 3. Incubation of tumor cells with glucose caused an increase in the contents of glucose-6-P plus fructose-6-P, fructose-1,6-P₂, and triose phosphates. Accumulation of these metabolites has also been observed by other authors upon incubation of ascites tumor cells with sugar for shorter periods of time (34,36). However, addition of either glutamine or asparagine further increased the content of hexose monophosphates to about 70% over the control values, whereas the contents of fructose-1,6-P₂ and triose phosphates drastically diminished under the same conditions. This effect was apparent after incubation for 60 min and remained over the next 120 min. Hence, a crossover point was produced at the step catalyzed by phosphofructokinase evidencing a strong decrease in the flux through this reaction. Incubation in the presence of proline changed the contents of these intermediates in the same direction but to a much lesser extent, in agreement with the lower effect promoted by this amino acid on the operation of glycolysis. The levels of both

![Fig. 2. Effect of glutamine, asparagine, and proline on the release of 14C-labeled anions (A) and [U-14C]glucose consumption (B) by ascites tumor cells as a function of time. For details see legend for Fig. 1.](image)

![Fig. 3. Effect of glutamine, asparagine, and proline on content of selected glycolytic intermediates of ascites tumor cells as a function of time. Glucose-6-P and fructose-6-P were measured separately, but their values were added to simplify the figure. Triose-P indicates glyceraldehyde 3-phosphate plus dihydroxyacetone phosphate. Dashed lines have been drawn between the points obtained at zero time and 60 min, as the changes in the content of some of these intermediates in ascites cells are known to be not linear during the early minutes of incubation (34-36). Each point shows the mean ± S.E. of three to six separate experiments. Incubation conditions and other details were as described in the legend for Fig. 1.](image)
P-enolpyruvate and pyruvate increased after 60-180 min when glutamine or asparagine were added to the incubation medium; these changes were greater than the change produced by proline. The 4-fold increase in P-enolpyruvate over the control values may reflect an inhibition of pyruvate kinase related to the large decrease in the content of fructose-1,6-P₂, a potent allosteric activator of this enzyme (37). This is supported by the intracellular concentrations of fructose-1,6-P₂ and P-enolpyruvate (29 and 330 μM, respectively, after incubation with glutamine or asparagine, as calculated from Fig. 3), that agree with the Kₘ value of 25 μM for fructose-1,6-P₂ at 250 μM P-enolpyruvate reported for pyruvate kinase from ascites tumor (37). This hypothesis could also explain the lower increase in P-enolpyruvate observed after incubation with proline, as this amino acid produced a smaller decrease in fructose-1,6-P₂. The pyruvate that accumulated about 12-fold above the control, after incubations with glutamine or asparagine for 60-180 min, must therefore have originated from the amino acid.

The Mechanism of Phosphofructokinase Inhibition—One may think that the marked inhibition of phosphofructokinase and glycolysis promoted by glutamine and asparagine was due to the concomitant decrease in the content of fructose-2,6-P₂. However, proline affected the operation of glycolysis very little in spite of being the most potent amino acid in decreasing fructose-2,6-P₂, and isoleucine, which also greatly diminished the levels of fructose-2,6-P₂, had no effect on the glycolytic flux (see above). Furthermore, as shown in Fig. 4, the maximal charge found in the concentration of fructose-2,6-P₂, from 6 μM after 180 min of incubation of cells with 5 mM glucose to about 2 μM after incubation in the presence of sugar plus 1 mM glutamine or asparagine, produced only a 10% decrease in phosphofructokinase activity assayed under conditions near to those prevailing in vivo when glycolysis was most strongly inhibited, i.e., upon incubation of cells with either of these amino acids from 120 to 180 min. Neither glutamine, asparagine, nor proline (1 mM) had any effect on the activity of isolated ascites tumor phosphofructokinase, and this activity was not changed in gel-filtered extracts of cells incubated with any of these amino acids (data not shown).

We measured the levels of other phosphofructokinase effectors, including P-enolpyruvate, Pₐ, the intracellular H⁺, citrate, and adenine nucleotides in ascites cells incubated with the amino acids to find out if any of these might be responsible for the observed inhibition of glycolysis. Whatever the signal might be, it should be generated during incubation with either glutamine or asparagine but to a much lesser extent or not at all with proline. Fructose-1,6-P₂ and NH₄⁺ were not considered since the activity of the ascites tumor isozyme is not sensitive to the former (42) and the latter, also an activator, is known to accumulate upon incubation of these cells with glutamine or asparagine (23). P-enolpyruvate is known to inhibit ascites tumor phosphofructokinase (42). However, the observed increase of this metabolite may contribute very little to the enzyme inhibition, since its highest intracellular concentration in the presence of amino acid, 0.33 mM, was below the reported Kᵣ value, 0.7 mM (42). The content of Pₐ, a positive effector, decreased from 3.3 ± 0.1 μmol/g at zero time to 1.1 ± 0.1 μmol/g after 180 min of incubation with 5 mM glucose, but this decrease was not altered significantly upon addition of any of the three amino acids to the incubation medium (data not shown). The calculated intracellular pH remained constant at about pH 7.2 after incubations up to 180 min either in the absence or the presence of any amino acid (data not shown). Fig. 5 shows that citrate, a negative effector, increased two to three times after incubation with amino acid, but no significant difference was observed between the three amino acids. Fig. 5 also shows the changes found in adenine nucleotides. Incubation of cells with glucose resulted in a 40% decrease in the content of ATP after 120 min, no significant change of ADP, and a decrease of about 60% AMP, the levels of total adenine nucleotides being diminished. A rapid and eventually reversible decrease of ATP and total adenine nucleotides upon addition of glucose to starved ascites tumor cells has been observed by others (34, 36, 43). When glutamine, or asparagine, were added to the incubation medium, the content of ATP was maintained considerably higher, over 80% after 120 min of incubation, AMP levels further decreased to about 24% of the control.

![Fig. 4. Effect of fructose-2,6-P₂ on the activity of partially purified ascites tumor phosphofructokinase at physiological concentrations of substrates and effectors. The assay contained 0.16 mM fructose-6-P, 2.8 mM MgATP, 7 mM Pi, 0.05 mM AMP, 0.5 mM NH₄⁺, 0.92 mM magnesium citrate, and 0.34 mM P-enolpyruvate. These concentrations corresponded to the intracellular concentrations determined after incubation of cells in the presence of 1 mM glutamine for 120 min and were calculated from data shown in Figs. 3 and 5. The Pₐ concentration was determined under similar conditions. The NH₄⁺ concentration was taken from Ref. 23. The assay was carried out at pH 7.2, as this was the intracellular pH value measured under these conditions. 10% PEG was added to the reaction mixture to increase the local enzyme concentration for a better simulation of the situation in vivo, since phosphofructokinase from ascites tumor and several other sources is known to exhibit concentration-dependent activity (38). Arrows indicate the intracellular concentration of fructose-2,6-P₂ determined after incubation of cells without (6 μM) and with 1 mM glutamine (2 μM) for 180 min, as calculated from data shown in Fig. 1.](image-url)

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2 The proportion of free fructose-2,6-P₂ available for phosphofructokinase in ascites cells is unknown. Nevertheless, it is likely to be substantially higher than that reported in liver by Hue et al. (39), since fructose-1,6-bisphosphatase activity, the protein that accounts for more than 80% of the total binding capacity of this tissue for fructose-2,6-P₂, is two orders of magnitude lower in ascites cells, 0.25 ± 0.01 unit/g according to Gumaa and McLean (34) versus 15 units/g in liver (39 and references therein). Moreover, the activity of fructosephospho-2-kinase/fructose-2,6-bisphosphatase, which contributes to the binding of this compound to a much smaller extent (39), is also lower in ascites cells, we measured 0.27 ± 0.05 milliunits/g for the kinase and 1.7 ± 0.2 milliunits/g for the bisphosphatase in these cells, versus 3.2 milliunits/g (40) and 10 milliunits/g (41), respectively, reported in liver.
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An increase of ATP from 1.4 to 2.8 mM, the intracellular concentrations calculated after 120 min of incubation without and with the inhibitory amino acid, respectively, resulted in a 30% decrease in the activity of asites tumor phosphofructokinase assayed at the concentrations of fructose-6-P and relevant effectors determined after incubation with glutamine or asparagine, as described in the legend for Fig. 4 and in the presence of 2 mM fructose-2,6-P_2. By the same token, the decrease of AMP from 0.15 to 0.04 mM, observed under the same conditions caused a 50% decrease of enzyme activity.

Hence, the individual change in ATP and AMP concentration may elicit a modification of phosphofructokinase activity greater than that promoted by other effectors, such as fructose-2,6-P_2 or P-enolpyruvate, although not enough to fully account for the decrease of the glycolytic flux found in vivo. This is not surprising, since phosphofructokinase is well known to be a multimodulated enzyme the net activity of which results from the balance between its various regulatory signals (38, 45, 46). Therefore, we assessed the joint effect of metabolites on the enzyme activity under conditions comparable to those of tumor cells during the observed inhibition of glycolysis. As shown in Table II, the reaction rate at concentrations of substrates and effectors determined after incubation of cells for 120 min in the presence of glutamine was only 29% of that measured under the conditions of the control. Thus, the change in metabolite levels may account for practically 90% of the inhibition of glycolysis found upon incubation of cells with glutamine or asparagine since the glycolytic flux decreased about 80% under these conditions. In contrast, under the conditions of incubation in the presence of proline the enzyme activity was as much as 65% of the control, in agreement with the lower effect produced by this amino acid on the operation of glycolysis.

*Studies on the Mechanism of the Decrease of Fructose-2,6-P_2 Content by Amino Acids—6-Phosphofructo-2-kinase and fructose-2,6-bisphosphatase, the enzymatic activities involved in the synthesis and degradation of fructose-2,6-P_2, respectively, have been identified and partially characterized in asites tumor cells (8). No significant change in these activities was observed when they were measured in gel-filtered extracts of cells incubated from 60 to 180 min with any of the amino acids that induced a fall in the content of fructose-2,6-P_2 (data not shown). This suggests that the decrease of this compound was not mediated by a stable modification of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase but by the change in the intracellular concentration of some of the metabolites that regulate these activities. In this case, the changes responsible should be apparent upon incubation of cells with either glutamine, asparagine, or proline, since the three of them promoted a similar decrease in fructose-2,6-P_2 content (Table I). This condition was not met by several of the potential regulators already determined during the incubations, such as P_i, fructose-6-P, P-enolpyruvate, or ATP.

![Graph showing the effect of glutamine, asparagine, and proline on content of citrate and adenine nucleotides of ascites tumor cells as a function of time.](image)

Fig. 5. Effect of glutamine, asparagine, and proline on content of citrate and adenine nucleotides of ascites tumor cells as a function of time. Each point shows the mean ± S.E. of three to six separate experiments. Incubation conditions and other details were as described in the legends for Figs. 1 and 3.

Values, ADP was unaffected, and the adenine nucleotide pool increased. In contrast, ATP content scarcely changed in the presence of proline, as compared with the control, and the decrease of AMP was substantially smaller. The calculated [ATP]/[ADP] × [P_i] ratio or cytosolic phosphorylation potential (44) did not significantly change after 120 min of incubation with proline (1004 ± 23 M\(^{-1}\)) versus the control (924 ± 73 M\(^{-1}\)), whereas it increased about 2-fold after addition of glutamine (1827 ± 47 M\(^{-1}\)) or asparagine (1805 ± 63 M\(^{-1}\)). This is consistent with the observed inhibition of phosphofructokinase.

If the free cytosolic [ADP] is assumed to be about 20-fold lower than measured [ADP] content, as suggested by Veech et al. (44) in mitochondrial-containing tissues, then the calculated values for the cytosolic phosphorylation potential would be 20 times higher.
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However, citrate, an inhibitor of 6-phosphofructo-2-kinase from liver (47, 48), muscle (47), and yeast (49), increased in a similar way after incubation of cells with any one of the three amino acids (Fig. 5). We found that citrate inhibited the ascites tumor enzyme with a $K_i$ value of 0.45 mM, its effect being to decrease the $V_{max}$ without significantly affecting the $K_m$ (data not shown). This $K_i$ value was within the range of the intracellular concentration of citrate in ascites tumor cells. An increase in the concentration of citrate from 0.4 to 0.9 mM, the values obtained after 120-min incubations without and with amino acid, respectively, produced a 40% decrease in 6-phosphofructo2-kinase activity measured at physiological concentrations of substrates and $P_i$. Furthermore, this activity decreased 51-55% when determined at the concentrations of fructose-6-P, ATP, $P_i$, and citrate calculated after 120 min of incubation of cells in the presence of either of the three amino acids compared with the control experiment. Therefore, the changes in the levels of citrate may play an important role in accounting for the observed diminution of fructose-2,6-P$_2$.

The Effect of Inhibiting Transamination on the Metabolic Changes Induced by Amino Acids—The phenomena described above were specifically produced by certain amino acids, which therefore must exhibit some common metabolic feature. There is no structural relation between the R groups of all of them. However, transamination with $\alpha$-ketoglutarate is involved in their degradation. This reaction is an intermediate step in the utilization of asparagine, aspartate, and isoleucine. Glutamate, a final product of the catabolism of glutamine and proline, is fed into the citric acid cycle to generate ATP via glutamate dehydrogenase or transamination. To investigate the role of transamination, we studied the effect of amino-oxyacetate, a potent inhibitor of transaminases in general (50), on relevant metabolic changes induced by amino acids in ascites cells (Fig. 6). Amino-oxyacetate (1 mM) prevented the effects of glutamine, asparagine, or proline, as shown by the decrease in the release of $^{14}$C-labeled anions and of glucose consumption, fructose-1,6-P$_2$, triose phosphates, and fructose-2,6-P$_2$ and the accumulation of hexose monophosphates, ATP, and citrate.

**DISCUSSION**

Glutamine and asparagine caused an 80% inhibition of glycolysis in ascites tumor cells which was specific and dose-dependent. This interaction was mediated by a strong inhibition of phosphofructokinase activity (Fig. 3), which can be accounted for by changes in various allosteric effectors (Table II). The major change responsible for this phenomenon is the increase of ATP and the decrease of AMP produced in the presence of glutamine or asparagine (Fig. 5). These amino acids as well as several others brought about a marked fall of fructose-2,6-P$_2$ content (Fig. 1 and Table I), which, although specific (Table I) and dose-dependent, was of less significance, since it modified very little the activity of phosphofructokinase assayed under physiological conditions (Fig. 4). On the other hand, some of the amino acids that decreased fructose-2,6-P$_2$ had little or no effect on the rate of glycolysis. Inhibi-

### Table II

Activity of partially purified ascites tumor phosphofructokinase at concentrations of substrates and effectors present in ascites tumor cells under various incubation conditions

<table>
<thead>
<tr>
<th>Condition of incubation</th>
<th>Conc. of substrates and effectors</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mM glucose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+1 mM glutamine</td>
<td>Fructose-6-P 0.10  ATP 1.4 AMP 0.15</td>
<td>0.006 0.07 0.40 0.147 ± 0.008 100</td>
</tr>
<tr>
<td>+1 mM proline</td>
<td>Fructose-6-P 0.16  ATP 2.8 AMP 0.04</td>
<td>0.002 0.34 0.92 0.043 ± 0.005 29</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 6.** Effect of amino-oxyacetate on the release of $^{14}$C-labeled anions, the consumption of [U-$^{14}$C]glucose, and the content of selected metabolites in the presence of either glutamine, asparagine, or proline in ascites tumor cells. Cells were incubated for 180 min with (black bars) or without (white bars) 1 mM amino-oxyacetate in the presence of 5 mM glucose and 1 mM amino acid as indicated. Results show the mean ± S.E. of three to six separate experiments. Differences between incubations with glucose and glucose plus any other addition that are statistically significant are indicated by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.005$).
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The role of phosphofructokinase as the step controlling the flux through glycolysis in ascites tumor cells has been questioned (51) on the basis of published data showing accumulation of the reaction product, fructose-1,6-P_2, in cells incubated with glucose as the sole energy source. The authors related the capacity of the tumor to metabolize large amounts of glucose to the lack of regulation at the phosphofructokinase level. This view is not supported by our results. Fructose-1,6-P_2 accumulated in the control experiment, pointing to a rate-limiting step further down the pathway under these conditions. However, this metabolite pattern was completely reversed when cells were incubated with glucose plus glutamine or asparagine, which in fact resembles a more physiological situation. The further increase of glucose-6-P and fructose-6-P and the large decrease of fructose-1,6-P_2 and triose phosphates induced by the amino acids (Fig. 3) demonstrate the strong limitation to the glycolytic flux imposed by the phosphofructokinase reaction.

It is noteworthy that the metabolic changes induced by asparagine were remarkably similar to those promoted by glutamine, indicating that the former is as efficiently metabolized as the latter. Both amino acids contributed to maintain high levels of adenylates, in agreement with data reported by others (11, 52, 53) showing that the oxidation of glutamine by these tumor cells results in a significant increase of ATP and the total adenine nucleotide pool. This is apparently caused by an activation of the synthesis of ATP and citrate to contribute to the observed inhibition of glycolysis and the fall of fructose-2,6-P_2, respectively. It is tempting to suggest that the reason why other amino acids were without effect on glucose metabolism may rest on their limited use as energy sources by tumor cells.

In conclusion, the results presented here clearly show that the glycolytic function in ascites tumor cells is dependent on glutamine and asparagine utilization, hence, allowing a coordinated operation for these two important aspects of their energetic metabolism. It would be of interest to determine whether this interaction is restricted to ascites tumor cells or it also occurs in other types of cancer and rapidly dividing cells.

Acknowledgment—We thank Dr. C. Gancedo for critical reading of the manuscript.

REFERENCES

Inhibition of Glycolysis by Amino Acids

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